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14. ABSTRACT Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 liganded AR. We have now published our data showing the AR N-terminus makes a critical contribution to NCoR binding (Hodgson, et al. 2005). Our recent not yet published results show that the N1 CoRNR box of NCoR mediates the interaction between NCoR and the AR ligand binding domain. Aim 2 is to determine whether NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers in vivo. Our recent data have shown that RU486 can markedly repress the growth of androgen independent C4-2 prostate cancer cells, and that this correlates with downregulation of AR regulated genes. Taken together, these results reflect substantial progress towards determining the structural basis for AR-NCoR interaction (Aim 1) and determining whether this interaction can be exploited to treat prostate cancer (Aim 2).					
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INTRODUCTION

The androgen receptor (AR) plays a central role in prostate cancer (PCa) and androgen ablation therapy is the standard systemic therapy for metastatic PCa, but most patients relapse with an aggressive stage of the disease termed hormone refractory or androgen independent PCa. The AR and androgen regulated genes are still expressed in androgen independent PCa, indicating that the AR remains as a therapeutic target for higher affinity pure antagonists. However, such drugs that can compete with dihydrotestosterone (DHT) for AR binding have not been developed. An alternative is the development of drugs that enhance AR recruitment of nuclear receptor corepressors (NCoR or SMRT), as such drugs could actively repress AR regulated genes. We have shown that the DHT liganded AR binds NCoR, and that this binding can be markedly enhanced by RU486 (mifepristone), a steroidal antagonist of the progesterone and glucocorticoid receptors. **Our hypothesis is that enhancement of the AR-NCoR interaction is a therapeutic approach for the treatment of PCa, including advanced androgen independent PCa.** The RU486 data provide a “proof of principle” that the AR-NCoR interaction can be enhanced, and suggest a novel mechanism for antagonist binding that may be valuable in the further development of high affinity AR antagonists. **Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 liganded AR. Aim 2 is to test the hypothesis that NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers *in vivo*.**

BODY

We present below our progress toward the specific aims.

AR N-terminus is required for AR-NCoR interaction. We have now published data showing that the AR N-terminus makes a critical contribution to AR-NCoR binding, and this work is detailed in the attached manuscript (Hodgson et al., 2005).

NCoR binding to the RU486 liganded AR is mediated by lysine 720 at the C-terminus of helix 3 in the AR LBD. We used the crystal structure of the antagonist liganded PPAR γ LBD bound to a SMRT derived CoRNR box peptide (core sequence -LEAIIRKAL-) as a model for understanding how NCoR might bind to the AR. A conserved lysine residue at the C-terminal end of helix 3 (corresponding to K720 in the AR) anchors the CoRNR box peptide by forming 3 hydrogen bonds with the carbonyls of the C-terminal leucine (residue 9 in the CoRNR box) and its flanking amino acids. This same lysine similarly anchors the C-terminal leucine in the coactivator LXXLL motif and also the AR N-terminal FQNLF, forming one end of a charge clamp. The CoRNR box forms three helical turns, with the leucines at positions 1 and 9, and the isoleucine at position 5, forming a hydrophobic face (underlined above) that binds to helix 3. Another face of the CoRNR box helix is formed by glutamic acid at position 2 and arginine at position 6, which form a strong intramolecular hydrogen bond and also hydrogen bond with N303 and K310 in helix 4 of PPAR γ , which correspond to D731 and Q738 in helix 4 of the AR, respectively.

We first mutated K720 at the C-terminus of helix 3 in the AR LBD to address whether NCoR binding was disrupted, as would be predicted from the PPAR γ structure. As expected, a K720A mutation impaired transactivation by the DHT liganded AR, although the effect was modest (perhaps due to strong hydrophobic interactions mediated by the phenylalanines in the AR N-terminal FQNLF peptide) (Fig. 1). Significantly, the K720A mutation markedly impaired interaction of the NCoR C-terminus with the RU486 liganded AR (Fig. 1), consistent with this residue anchoring a CoRNR box.

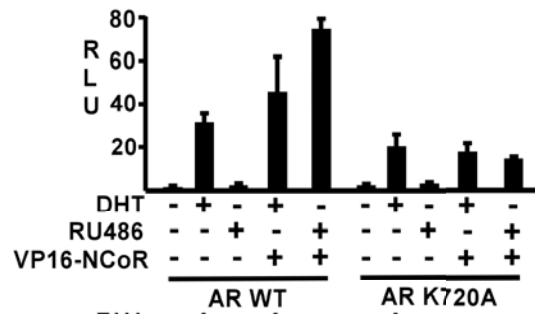


Fig. 1. NCoR binding to RU486 liganded AR is impaired by K720A mutation. CV1 cells were transfected with wild-type or K720A mutant AR, VP16-NCoR, ARE₄-luciferase reporter, and control pRL-CMV (*Renilla*) reporter. They were then treated for 24 hrs with 10 nM DHT, 10 nM RU486, or no hormone. Firefly versus *Renilla* luciferase activities were measured from triplicate samples. Relative light units (RLU) reflect normalized firefly/*Renilla* (+SD).

NCoR binding to the RU486 liganded AR is mediated by the C-terminal N1 CoRNR box in NCoR. As NCoR deletion mutants indicated that both N2 and N1 were required for robust NCoR binding to the RU486 liganded full length AR, we next examined a series of conservative mutations in the N1 and N2 CoRNR boxes in the Gal4DBD-NCoR(1806-2454) vector, encoding all three receptor interacting domains and referred to as Gal4DBD-NCoR(N3-N1). As shown previously, VP16-AR binding to wild-type Gal4DBD-NCoR(N3-N1) was markedly stimulated by RU486 (Fig. 2A). Strikingly, a double alanine mutation in N1 (LEDII to LEDAA) abrogated binding, while the comparable mutation in the N2 CoRNR box (ICQII to ICQAA) had no effect. As controls to confirm that the N1(AA) protein was expressed and functional, we demonstrated equivalent expression of all three proteins by immunoblotting (not shown) and that they all interacted with the unliganded TR β , which binds NCoR through the N3 CoRNR box (Fig. 2B).

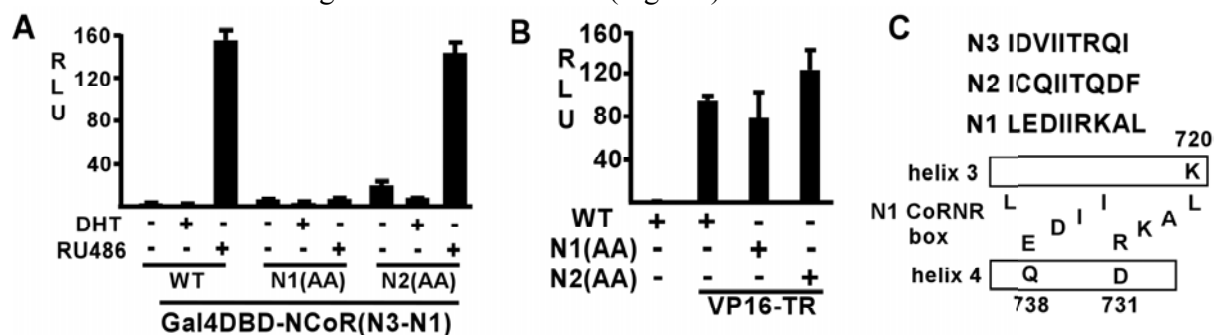


Fig. 2. N1 CoRNR mediates binding to RU486 liganded AR. A, CV1 cells were transfected with wild-type or mutant Gal4DBD-NCoR(N3-N1), VP16-AR, pG5-luciferase and control pRL-CMV reporters, and treated with 10 nM DHT or RU486 in steroid depleted medium, as indicated. B, cells were transfected as in A, but with VP16-TR β and cultured in steroid and T₃ depleted medium. C, CoRNR box sequences and diagram of N1 CoRNR box interactions with AR helices 3 and 4.

Significantly, the sequence of the extended N1 CoRNR box (LEDIIRKAL) has a basic arginine at position 6, as well as the glutamic acid at position 2, which would form one face of the CoRNR box helix interacting with helix 4 in the AR LBD. As diagrammed in figure 2C, strong interactions between these residues and aspartic acid (position 731) and glutamine (position 738) in helix 4 of the AR LBD may stabilize this interaction. Neither the N3 or N2 CoRNR boxes have both acidic residues at position 2 and basic at position 6, although these are present in the N1 box of SMRT. Site directed mutagenesis of these sites is being done to assess their importance and potentially generate additional mutants for functional studies. In any case, the N1(AA) mutant will be used in Aim 1 for functional studies correlating AR-NCoR binding and transcriptional repression.

RU486 represses proliferation of LNCaP derived "androgen independent" C4-2 cells. We showed previously that RU486 strongly repressed growth of LNCaP cells in medium containing androgens (RPMI-1640 with 10% FBS) and could further suppress the growth of LNCaP cells grown in steroid hormone depleted medium (RPMI-1640 with 10% charcoal dextran stripped FBS), but this latter suppression was only modest (approximately 20%, data not shown). To determine whether RU486 would suppress "androgen independent" PCa cells, we examined the LNCaP subline C4-2, derived from a LNCaP xenograft that relapsed after castration. The C4-2 cells express high levels of AR and appear to have reactivated AR by unclear mechanisms that are not blocked by antiandrogens such as bicalutamide. Indeed, as shown in figure 3, C4-2 cells proliferate well in steroid hormone depleted medium (S-phase ~19%) versus 3-5% for the parental LNCaP cells (not shown). Significantly, proliferation of the C4-2 cells is markedly suppressed within 24 hours by 10 nM RU486 (S-phase ~3%), with a corresponding increase in G₀/G₁ cells (Fig. 3). This cell cycle arrest is similar to what occur after androgen withdrawal in LNCaP cells, suggesting that RU486 may be repressing certain androgen responsive growth promoting genes that are reactivated in C4-2.

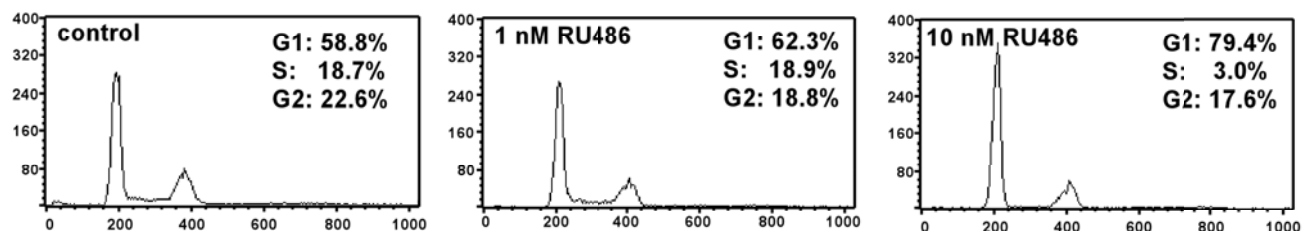


Fig. 3. RU486 suppresses proliferation of androgen independent C4-2 cells. C4-2 cells were cultured in steroid hormone depleted medium (10% CDS-FBS) and then treated with vehicle (control) or RU486 as indicated for 24 hrs. Cell cycle distribution was determined by flow cytometry using propidium iodide, with identical results in a replicate experiment.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that AR N-terminus is critical for AR-NCoR interaction.
2. Demonstrated that lysine 720 in AR LBD is critical for binding.
3. Identified the N1 CoRNR box as mediating binding to the AR LBD.
4. Demonstrated that RU486 could suppress the androgen independent C4-2 prostate cancer cell line.

REPORTABLE OUTCOMES

One new manuscript published (attached) (Hodgson et al., 2005).

CONCLUSIONS

We have made substantial progress towards determining the structural basis for AR-NCoR interaction (Aim 1) and determining whether this interaction can be exploited to treat prostate cancer (Aim 2).

REFERENCES

Hodgson,M.C., Astapova,I., Cheng,S., Lee,L.J., Verhoeven,M.C., Choi,E., Balk,S.P., and Hollenberg,A.N. (2005). The androgen receptor recruits nuclear receptor CoRepressor (N-CoR) in the presence of mifepristone via its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists. *J. Biol. Chem.* 280, 6511-6519.

APPENDICES

Manuscript attached (Hodgson et al., 2005).

The Androgen Receptor Recruits Nuclear Receptor CoRepressor (N-CoR) in the Presence of Mifepristone via Its N and C Termini Revealing a Novel Molecular Mechanism for Androgen Receptor Antagonists*

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The androgen receptor (AR) activates target gene expression in the presence of agonist ligands via the recruitment of transcriptional coactivators, but recent work shows that overexpression of the nuclear corepressors NCoR and SMRT attenuates this agonist-mediated AR activation. Here we demonstrate using NCoR siRNA and chromatin immunoprecipitation that endogenous NCoR is recruited to and represses the dihydrotestosterone (DHT)-liganded AR. Furthermore this study shows that NCoR and coactivators compete for AR in the presence of DHT. AR antagonists such as bicalutamide that are currently in use for prostate cancer treatment can also mediate NCoR recruitment, but mifepristone (RU486) at nanomolar concentrations is unique in its ability to markedly enhance the AR-NCoR interaction. The RU486-liganded AR interacted with a C-terminal fragment of NCoR, and this interaction was mediated by the two most C-terminal nuclear receptor interacting domains (RIDs) present in NCoR. Significantly, in addition to the AR ligand binding domain, the AR N terminus was also required for this interaction. Mutagenesis studies demonstrate that the N-terminal surface of the AR-mediating NCoR recruitment was distinct from tau5 and from the FXXLF motif that mediates agonist-induced N-C-terminal interaction. Taken together these data demonstrate that NCoR is a physiological regulator of the AR and reveal a new mechanism for AR antagonism that may be exploited for the development of more potent AR antagonists.

The androgen receptor (AR),¹ a member of the steroid/nuclear receptor superfamily, plays a critical role in normal male

development, including the development of the prostate gland. In addition, AR action plays a fundamental role in the development and progression of prostate cancer (1–3). Prostate cancers are initially androgen responsive such that targeted therapies aimed at lowering circulating androgen levels are the treatment of choice for metastatic disease. In most cases, however, the disease becomes progressive and unresponsive to androgen ablation therapies. This progressive stage of the disease, referred to as hormone refractory or androgen-independent prostate cancer, is generally heralded by the re-expression of androgen-regulated genes such as prostate-specific antigen (PSA). AR gene amplification or mutations may contribute to this re-expression of androgen-regulated genes, but it occurs mainly through undefined molecular mechanisms that allow for AR signaling in the absence of ligand or at reduced systemic androgen levels (4–8). Thus, new approaches to silence AR signaling may have important therapeutic ramifications for the therapy of both early and late stage prostate cancer.

Members of the steroid receptor superfamily signal in a similar fashion based on their structural similarity (9). The addition of an agonist ligand leads initially to the DNA binding of the receptor, followed by the ordered recruitment of both transcriptional coactivators and other mediators to the ligand binding domain. This leads to histone modifications including acetylation, and finally to transcriptional activation (10). In contrast, in the presence of a hormone antagonist, steroid receptors fail to recruit coactivators. Moreover, certain antagonists preferentially stimulate the recruitment of nuclear receptor corepressors to target promoters, which in turn recruit a multiprotein complex that leads to histone deacetylation and transcriptional repression (11–14).

The AR is structurally similar to other steroid receptors in that it is recruited to target elements as a homodimer and contains a high affinity, steroid-specific ligand binding domain. However the AR differs in the molecular mechanisms by which it recruits both coactivators and corepressors. Unlike other family members, the ligand-binding domain of the AR preferentially recruits its own AF-1 domain via an N-terminal FXXLF motif, which interacts strongly with the hydrophobic cleft of the LBD created by ligand binding (15–19). The subsequent recruitment of coactivators, in particular the SRC family, is then mediated primarily by the AF-1 domain rather than by the LBD (18, 20–23). In addition, we and others (24–28) have shown that the agonist-liganded AR can recruit the corepressors NCoR and SMRT, suggesting that agonist-dependent activation of the AR may be dependent upon the relative levels of these corepressors *versus* coactivators.

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¹ The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; PSA, prostate-specific antigen; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; LBD, ligand binding domain; RLU, relative light units; RID, receptor interacting domains; HA, hemagglutinin; DBD, DNA binding domain; CMV, cytomegalovirus; NCoR, nuclear corepressor; siRNA, small interfering RNAs.

Besides the physiological agonists testosterone and DHT, the AR can also interact with many other steroidal or nonsteroidal drugs that function as relatively pure antagonists (such as hydroxyflutamide and bicalutamide) or as partial agonists (29). Bicalutamide, which is widely used for prostate cancer treatment, can stimulate the AR to bind DNA, but fails to recruit coactivators and can mediate the recruitment of NCoR to the androgen-regulated PSA gene, indicating that corepressor recruitment may contribute to antagonist activity (30–32). Nonetheless, bicalutamide has limited efficacy in the advanced androgen-independent stage of prostate cancer, and other AR antagonists are similarly ineffective at this stage of the disease (33). As enhancement of corepressor recruitment to the AR may be an effective approach for blocking AR signaling in prostate cancer, this study further examines corepressor recruitment by AR agonists and antagonists.

Loss of function experiments were carried out initially using NCoR siRNA, in conjunction with chromatin immunoprecipitation (ChIP). These confirmed that endogenous NCoR could negatively regulate the activity of the DHT-liganded AR, and indicated that AR activity may be regulated by the relative levels of NCoR and coactivators. Although multiple other ligands could mediate AR-NCoR interaction, the AR partial agonist RU486 (mifepristone) functioned uniquely, at nanomolar concentrations, as a strong enhancer of this interaction. Significantly, while NCoR RIDs and the AR LBD contributed to AR-NCoR binding, this was markedly enhanced by a further interaction with the AR N terminus via a site that was independent of the N-terminal FXXLF motif. These results demonstrate that NCoR is a biological regulator of AR action, and identify a new role for the AR N terminus in the AR-NCoR interaction. Moreover, the marked enhancement of the AR-NCoR interaction by RU486, but not by AR antagonists currently in clinical use, indicates that this interaction is a target for the development of new potent AR antagonists.

MATERIALS AND METHODS

Plasmids and Reagents—Expression vectors for AR (pSVARo) ER α (pcDNA-ER α), NCoR (PKCR2-NCoR), and SRC1 (pSG5-SRC1) have been described previously (24, 34, 35). The NCoR vector (pKCR2-NCoRc) was referred to previously as NCoRI and encodes the C-terminal amino acids 1574–2454 of NCoR (numbering is based on murine NCoR). VP16-NCoRc encodes the three NCoR RIDs from NCoRc fused to the VP16 transactivation domain in the AASVVP16 vector (amino acids 1806–2454). Additional fusion protein constructs with VP16 were constructed in the pACT vector, while fusions to the Gal4 DNA binding domain were in the pBind vector (Promega, Madison, WI). The VP16 fusions with AR include VP16-AR (encoding the full-length AR), VP16-ARDBD-LBD (encoding amino acids 501–919), VP16-ARNTD (amino acids 1–500), VP16-ARLBD (amino acids 661–919), and have been described (24). Additional NCoR fragments fused to VP16 were generated by ligating the indicated PCR-amplified regions (numbering is based on the murine NCoR sequence) into pACT with a C-terminal HA tag. GST-NCoR (N2N1) contains amino acids 2063–2300 of hNCoR fused to GST in PGEX4T1 and has been described previously (36, 37). The AR mutant F23A was kindly provided by E. Wilson. The mutants LF26,27AA and ARdel23–27 were generated from pSVARo using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Gal4-NCoRc CoRRR box mutants were described previously (34, 35). The reporter constructs ARE₄-luciferase, containing four tandem copies of a synthetic ARE, and the estrogen response element (ERE₂)-luciferase containing two EREs have been described (24). pG5-luciferase, regulated by five tandem Gal4 binding sites, and pRL-CMV, a CMV promoter-regulated *Renilla* control, were from Promega. Dihydrotestosterone (DHT), estradiol (E₂), and RU486 were from Sigma and were used as 1:1000 stock solutions in ethanol.

Cell Culture and Transfection—CV1 and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells in 48-well tissue culture plates in DMEM containing 10% charcoal dextran-stripped fetal bovine serum (CDS-FBS, Hyclone) were cotransfected using Lipofectamine 2000 (Invitrogen). Cells were transfected with 50 ng of reporter vector, expression

vector, and coregulators (NCoR, NCoRc, SRC1) except where indicated, and with 1.25 ng of pRL-CMV *Renilla* vector for normalization. After 24 h the medium was replaced with fresh DMEM/10% CDS-FBS medium containing hormones or drugs at the indicated final concentrations. Following a further 24 h, firefly, and *Renilla* luciferase activities were assayed with the dual-luciferase assay system from Promega as per the supplier's instructions. All samples were in triplicate or quadruplicate and firefly luciferase activities were normalized for cotransfected *Renilla* activity. To assess expression of transfected proteins, lysates from replicate wells were prepared in 1% SDS, run on 8 or 10% SDS-PAGE, and immunoblotted with anti-HA (Covance) or anti-AR antibodies (Santa Cruz Biotechnology) (1:1000 dilutions incubated overnight at 4 °C), followed by horseradish peroxidase-conjugated anti-mouse or rabbit Ig secondaries (Promega) and ECL (Amersham Biosciences).

RNA Interference—Vectors expressing hairpin small interfering RNAs (siRNA) under the control of the mouse U6 promoter were constructed by inserting pairs of annealed DNA oligonucleotides into pBS/U6 plasmid (a gift of Y. Shi) at ApaI-EcoRI sites (38). The target sequence for NCoR was: 5'-GGGCTTATGGAGACCCTATGA-3'. To assess effects on AR transactivation, this siRNA plasmid (pU6-NCoR) or a control laminin siRNA plasmid (pU6-laminin) were cotransfected with the AR expression vector and the indicated reporters. To assess protein expression, cells in 6-well plates were transfected with 0.1–1.6 μ g of vector per well. At 48 h after transfection the cells were collected, and nuclear extracts were isolated (39). Nuclear proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred overnight to nitrocellulose membranes. Blots were probed with a 1:500 dilution of an affinity-purified anti-NCoR antiserum in Tris-buffered saline containing 5% nonfat milk and 0.05% Tween 20, followed by horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) at 1:1000 dilution (24). The blots were visualized using ECL Plus Western blotting detection system.

Chromatin Immunoprecipitation—LNCaP prostate cancer cells grown to ~80% confluence in 10-cm plates were switched to steroid hormone-depleted medium (RPMI 1640/10% CS-FCS) for 48 h and then exposed to 10 nM DHT for varying times. Plates were then rinsed with PBS and fixed for 10 min at room temperature with 1% formaldehyde in PBS. After rinsing twice with ice-cold PBS, cross-linking was terminated by scraping cells into 1 ml of 100 mM Tris, pH 9.4 and 10 mM dithiothreitol and incubating at 30 °C for 15 min. Cell pellets were then washed twice with PBS and resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and protease inhibitors). Chromatin was sheared to 300–1000 bp with three sequential 10 s pulses at 70% power with a microtip ultrasonic cell dismembrator (Fisher). Cell debris was pelleted at 10,000 \times g, and the supernatant was precleared with 10 μ g of sheared salmon sperm DNA, 20 μ l of non-immune sera, and 20 μ l of a 50% slurry of protein A-Sepharose. Immunoprecipitation was with 500 ng of AR N-terminal rabbit polyclonal antibody (PG-21, Upstate Biotechnology), affinity-purified NCoR C-terminal anti-peptide antibody (24) or a nonspecific control antibody, 2 μ g of sheared salmon sperm DNA, and 20 μ l of a 50% slurry of protein A-Sepharose. Precipitates were washed three times with 300 mM NaCl, 50 mM Tris, pH 8.0, 2.7 mM KCl, 0.05% Tween-20 and 1% deoxycholate. Three additional washes with 10 mM Tris, 1 mM EDTA were performed and then beads were eluted three times with 35 μ l of 1% SDS and 100 mM NaHCO₃ at 37 °C for 10 min each. Eluates were pooled and incubated at 65 °C overnight to reverse cross-links. Products were then purified with QiaQuick PCR purification spin resin (Qiagen, Valencia, CA) and 10% of the eluate was subjected to 50 cycles of PCR amplification with steps of 94 °C, 55 °C, and 72 °C for 1 min each. The primers for the p21 cyclin-dependent kinase promoter were 5'-AAGCTCCCTC-CTGGACCCAGA-3' and 5'-CAAGCCTCAGAGCATCAG-3'; and for the PSA promoter 5'-GAGAGCTAGCACTTGCTGT-3' and 5'-AGTTCTA-GTTTCTGGTCTCA-3'. PCR products were analyzed by gel electrophoresis and ethidium bromide staining in 4% agarose gels.

GST Pull-down Assays—GST-NCoR (N2N1) and GST alone were produced as described previously and purified using glutathione-agarose beads (36, 37) and there integrity was visualized on SDS-PAGE. To derive the indicated AR moiety, CV1 cells in 60-mm plates were transfected with either full-length AR, AR N-DBD, or D-LBD as described above. After 24 h, medium was replaced with fresh medium with or without 10 nM DHT or RU486. Cells were washed twice with ice-cold PBS and extracts prepared by scraping cells into 0.75 ml of lysis buffer (PBS, 5% glycerol, 0.05% Triton X-100 and protease inhibitors). Lysates were incubated on ice for 5 min and sonicated with two sequential 5-s pulses at 30% power with a microtip ultrasonic dismembrator (Fisher) and centrifuged at 4 °C to pellet insoluble material. Cell extracts were precleared with GST agarose beads for 2 h at 4 °C on a rotating wheel.

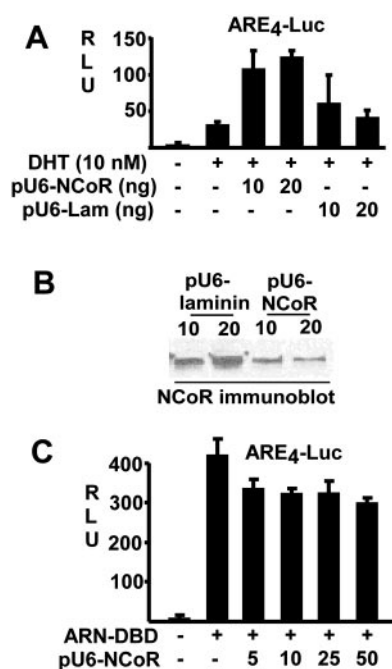


FIG. 1. Decreased nuclear NCoR levels enhance AR-mediated action. A, CV1 cells were transfected with AR, an ARE₄-luciferase reporter, and a control CMV-regulated *Renilla* luciferase plasmid (pRL-CMV), and plasmids expressing NCoR siRNA (pU6-NCoR) or control laminin siRNA (pU6-Lam), using Lipofectamine. Transfectants in steroid hormone-depleted medium (DMEM with 10% charcoal dextran-stripped fetal bovine serum) were stimulated for 24 h with DHT as indicated and luciferase *versus Renilla* luciferase activities were determined from triplicate samples (expressed as RLU \pm S.E.). B, nuclear extracts were prepared from CV1 cells transfected with either pU6-Lam or pU6-NCoR. Equal amounts were loaded onto SDS-PAGE. The resulting transferred proteins were probed with NCoR antiserum and exposed using chemiluminescence. C, CV1 cells were transfected as in A but with a plasmid encoding the AR N terminus and DBD, AR N-DBD.

Cleared lysates were incubated for 16 h with or without 10 nM DHT or RU486 at 4 °C on a rotating wheel in the presence of equal amounts of GST or GST-NCoR (N2N1) agarose beads. The beads were washed once with ice-cold lysis buffer and three times with ice-cold PBS and bound proteins were eluted by boiling in Laemmli sample buffer (Promega), run on 12% SDS-PAGE and blotted onto nitrocellulose membranes. To visualize AR proteins, Western blots were probed with either a polyclonal antiserum recognizing the first 20 or last 19 residues of hAR (Santa Cruz Biotechnology). Five percent of total cell lysates was loaded as input.

RESULTS

NCoR Down-regulation Enhances AR Transcriptional Activity—Previous studies have shown that transfected NCoR could repress the transcriptional activity of the DHT-liganded AR (24). RNAi was used to directly address whether endogenous NCoR functions as a negative regulator of AR transcriptional activity. CV1 cells were transfected with an AR expression vector and AR-regulated luciferase reporter gene, with or without plasmids encoding an NCoR or control siRNA expression vector (pU6-NCoR or pU6-laminin, respectively). As shown in Fig. 1A, AR transcriptional activity was augmented by the NCoR siRNA, but not the control siRNA plasmid. Consistent with this result, immunoblotting showed that NCoR protein expression was markedly down-regulated by the NCoR siRNA (Fig. 1B).

We showed previously that repression of AR transcriptional activity by transfected NCoR was dependent upon an interaction between NCoR receptor interacting domains and the AR LBD (24). Therefore, another control for nonspecific siRNA effects was to examine whether enhancement of AR activity by the NCoR siRNA was dependent on the AR LBD. In contrast to

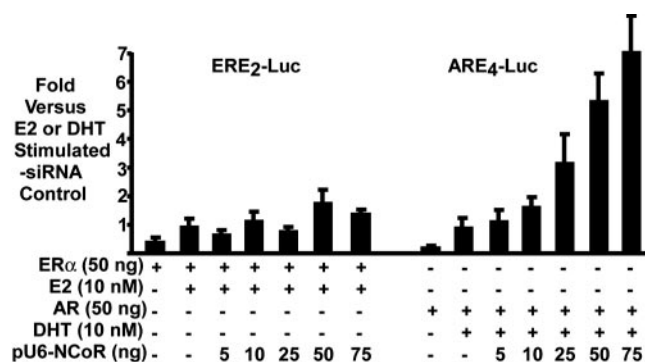


FIG. 2. NCoR levels do not influence ER-mediated action. CV1 cells were transfected with either ERα and an ERE-Luc reporter or the AR and the ARE₄-Luc reporter. All transfections also included the pRL-CMV control as well as the indicated amounts of pU6-NCoR. Cells were treated with the indicated ligands for 24 h, and luciferase *versus Renilla* luciferase activities were determined from triplicate samples (expressed as RLU \pm S.E.).

full-length AR, an AR expression vector with the LBD-deleted (AR N-DBD, encoding the N terminus and DBD) was constitutively active in the absence of DHT and was not stimulated by the NCoR siRNA (Fig. 1C).

As a further control, we examined the estradiol-liganded ERα. Whereas NCoR can interact with ERα when it is liganded by certain partial agonists, the estradiol-liganded ERα is not repressed by NCoR transfection. As shown in Fig. 2, the NCoR siRNA did not enhance the activity of the estradiol-liganded ERα, while AR activity was again markedly enhanced in the same experiment. Taken together, these data supported the conclusion that endogenous NCoR functions to suppress the transcriptional activity of the agonist-liganded AR.

NCoR Is Recruited to Androgen-regulated Genes by the Endogenous DHT-liganded AR in Prostate Cancer Cells—Although we have been able to markedly down-regulate NCoR expression by siRNA and enhance AR activity in CV1 and 293T cells (data not shown) efforts to substantially down-regulate NCoR protein in a prostate cancer cell line expressing endogenous AR have not yet been successful. Therefore, chromatin immunoprecipitation was used as an alternative approach to test the hypothesis that NCoR associates with the DHT-liganded endogenous AR and is recruited to AR-regulated genes. Androgen responsive LNCaP prostate cancer cells were grown in steroid hormone-depleted medium for 2 days, and were then pulsed with DHT. Chromatin was cross-linked with formaldehyde at varying times after the DHT pulse, and sheared chromatin was immunoprecipitated with anti-AR, anti-NCoR, or control antibodies. PCR was then used to assess AR and NCoR recruitment to AREs in the p21 cyclin-dependent kinase inhibitor promoter and PSA regulatory regions. As shown in Fig. 3A, DHT treatment led to the recruitment of AR to the p21 gene. Significantly, NCoR was not associated with the p21 ARE in the absence of DHT, consistent with the lack of AR binding, but became associated after DHT treatment. Interestingly, the binding of both AR and NCoR appeared to be transient based on this method, as has been reported previously for AR and ERα (31).

DHT similarly induced the association of both AR and NCoR with the PSA promoter (Fig. 3B), as well as the ARE in the PSA enhancer (data not shown). In this experiment the AR and NCoR association was not detected until 30–45 min, and it persisted for at least 2 h. More rapid and transient association of both AR and NCoR have been observed in other experiments (data not shown), and the basis for the variable recruitment kinetics are not yet clear. Nonetheless, a consistent correlation has been observed between AR and NCoR recruitment in re-

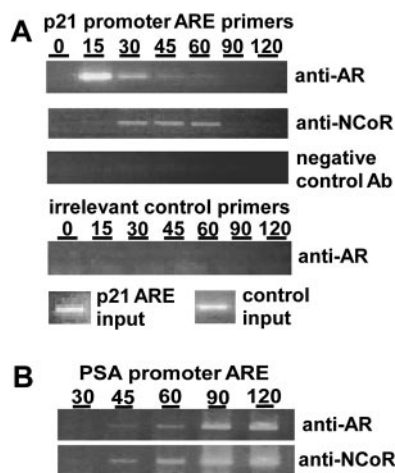


FIG. 3. NCoR is recruited to both the p21 and PSA promoters in the presence of DHT. A, LNCaP cells were treated with DHT for the indicated number of minutes, fixed, sheared, and immunoprecipitated with the indicated antibodies (rabbit anti-PDGF receptor antibody was used as a negative control). After reversing the cross-links the solubilized chromatin was subjected to PCR with primers spanning the p21 ARE or control primers for the human *cd1* gene. The resulting PCR products were resolved on ethidium bromide-stained agarose gels. Also shown is PCR from input chromatin used with the AR, NCoR, and control antiserum. B, an identical paradigm was used on precipitated DNA complexes treated as above and PCR-amplified with primers in the PSA promoter flanking the ARE.

sponse to DHT. It should be noted that previous studies have shown NCoR recruitment by antagonist (bicalutamide)-liganded AR, but not the DHT-liganded AR (31, 32). The basis for this difference is not clear, but may reflect the distinct affinity purified anti-NCoR antibodies used in this study. In any case, these data in conjunction with the siRNA results indicate that endogenous NCoR contributes to the regulation of AR transcriptional activity.

NCoR Represses SRC-1 Enhancement of AR Transcriptional Activity—We reported previously that NCoR repression of AR transcriptional activity was independent of the HDAC-interacting repressor domains in the NCoR N terminus, as repression was observed in the presence of trichostatin A (24). Moreover, AR could be repressed by the NCoR C terminus, encoding the receptor-interacting domains (RIDs), independently of the repressor domains in the NCoR N terminus. Significantly, repression was abrogated by mutations in the three NCoR CoRNR box motifs (LXXII) present in the RIDs, which are presumed to interact with helices 3–5 in the coactivator/corepressor binding site of the AR LBD. This suggested that repression might be caused by NCoR inhibition of the AR N-C-terminal interaction, as an LXXLL-like motif in the AR N terminus normally associates with the AR LBD and makes a major contribution to AR transcriptional activity. However, NCoR could also repress AR activation by partial agonists that do not mediate AR N-C-terminal interaction, and did not block the interaction between the AR N terminus and the DHT-liganded AR LBD in mammalian two-hybrid protein interaction assays (data not shown).

An alternative possible mechanism for AR repression by NCoR is inhibition of coactivator binding. Consistent with this mechanism, transfection of the NCoR C terminus strongly repressed AR coactivation by SRC-1 (Fig. 4A). Importantly, previous studies have shown that SRC-1 binding to the AR is mediated primarily by the AR N terminus, with little or no interaction between the NR boxes in SRC-1 and the AR LBD. This suggested that NCoR might be inhibiting SRC-1 binding by interacting directly with the AR N-terminal domain. This was tested by examining whether the NCoR C terminus, ex-

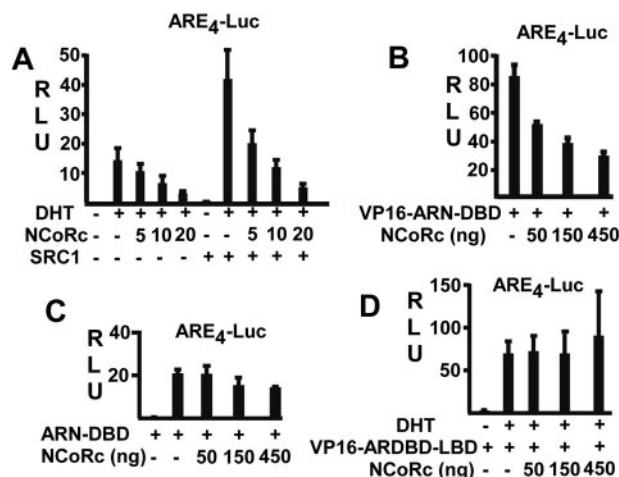


FIG. 4. NCoR blocks AR action via functional interactions with both AR N terminus and C terminus. A, CV1 cells were cotransfected with the AR, the ARE₄-Luc promoter and the pRL-CMV control. In addition cells were transfected with expression plasmids for NCoRc and either pSG5-SRC-1 or pSG5 alone. Transfectants in steroid hormone-depleted medium (DMEM with 10% charcoal dextran-stripped fetal bovine serum) were stimulated for 24 h with DHT as indicated. Luciferase *versus* Renilla luciferase activities were determined from triplicate samples (expressed as RLU \pm S.E.). B and C, CV1 cells were transfected as in A with either an expression plasmid for the AR N terminus and DNA-binding domain fused to VP16 (B, VP16-ARN-DBD) or the AR N terminus and DNA binding domain alone (C, AR N-DBD), the ARE₄-Luc-reporter and the pRL-CMV control with increasing amounts of NCoRc. D, CV1 cells were transfected as above with an expression plasmid for the AR DBD-LBD fused to VP16 (VP16-ARN-DBD-LBD), the ARE Luc-reporter and the pRL-CMV control with increasing amounts of NCoRc.

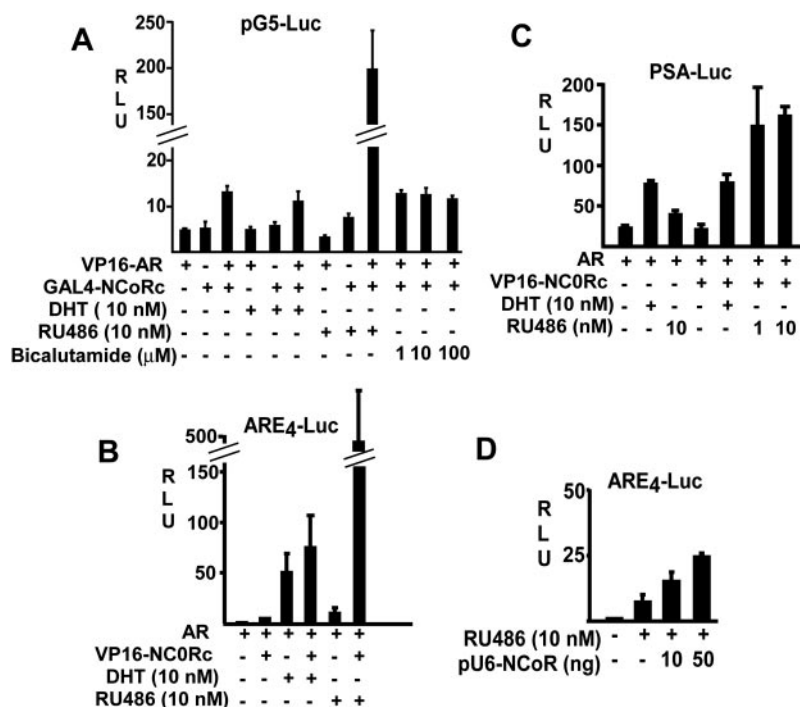
pressed at high levels, could repress the constitutive activity of the AR N-terminal domain.

CV1 cells were transfected with an AR N-DBD fragment (encoding the AR N terminus and DNA binding domain), which had high androgen-independent activity on an ARE reporter gene when fused to VP16 (Fig. 4B). Significantly, this activity was strongly repressed by cotransfection with NCoRc. It should be noted that this repression required the high level expression of transfected NCoRc, consistent with a relatively low affinity interaction and the failure of NCoR siRNA to enhance the activity of the AR N-DBD fragment (Fig. 1C). To confirm that this interaction was not dependent on the VP16 domain, we tested the AR N-DBD fragment alone on the ARE reporter. As shown in Fig. 4C, this construct had less activity than the VP16 fusion, but was also inhibited by cotransfected NCoRc. The repression seen in these experiments was specific as there was no effect on control CMV-regulated reporters (data not shown). Moreover, although NCoRc can interact with the AR LBD, it did not repress the transcriptional activity of the AR DBD and LBD fused to the VP16 transactivation domain (pACT-AR DBD-LBD) (Fig. 4D). Taken together, these results indicated that NCoR can interact with both the AR N and C termini, and that the relative levels of NCoR *versus* coactivator proteins may regulate AR transcriptional activity.

NCoR Interaction with AR Is Enhanced by RU486—To further assess the mechanisms mediating NCoR interaction with AR, and whether NCoR binding can be influenced by the conformation of the AR LBD, we next examined a series of AR partial agonists and antagonists. Of particular interest was whether enhanced NCoR binding might contribute to the effects of certain AR antagonists. To directly assay NCoR-AR binding, we carried out mammalian two-hybrid protein interaction assays using the NCoR C terminus fused to the Gal4 DBD (Gal4-NCoRc) and full-length AR fused to the VP16 trans-

FIG. 5. RU486 potentially enhances the recruitment of NCoR by the AR.

A, CV1 cells were cotransfected with expression plasmids for the full-length AR fused to VP16, and NCoRc to the Gal4-DNA binding domain as well as a multimerized UAS-Luc reporter (pG5-Luc) and the pRL-CMV control. The indicated ligands were added for 24 h, and luciferase *versus Renilla* luciferase activities were determined from triplicate samples. The data are expressed as RLU \pm S.E. B and C, CV1 cells were transfected with expression plasmids for the full-length AR and VP16-NCoRc in the presence of the ARE₄-Luc reporter or the PSA enhancer-promoter linked to luciferase. Cells were treated for 24 h with DHT or RU486, and luciferase *versus Renilla* luciferase activities were determined from triplicate samples. D, CV1 cells were cotransfected with the full-length AR expression vector, the ARE₄-Luc reporter and the pRL-CMV control with increasing amounts of pU6-NCoR. Cells were treated for 24 h with RU486 and luciferase *versus Renilla* luciferase activities were determined from triplicate samples.



activation domain (VP16-AR), in conjunction with the pG5-luciferase reporter (containing five tandem Gal4 elements recognized by the Gal4 DBD). Consistent with our previous data, a weak ligand-independent interaction between NCoR and AR could be detected, and this was not enhanced by DHT (Fig. 5A). Similarly to DHT, the NCoR-AR interaction was not substantially enhanced by a series of other partial agonists or antagonists, including bicalutamide (Fig. 5A), hydroxyflutamide, cyproterone acetate, estradiol, progesterone, or androstenedione (data not shown).

In contrast, the NCoR-AR interaction was markedly enhanced by RU486 (mifepristone) (Fig. 5A). RU486 was originally identified as a steroidal antagonist of the GR and PR, and more recent data have shown that NCoR interacts with the RU486-liganded GR and PR (13, 40–42). Two very recent reports also indicate that NCoR binds to the RU486-liganded AR (28, 43). The unique structural feature of RU486 is a bulky 11- β substitution that appears to interact with helix 3 and prevent the formation of the coactivator binding site. To confirm that RU486 could mediate NCoR recruitment to the unmodified AR, we examined the effect of a VP16-NCoRc fusion protein on AR transactivation of an ARE-regulated reporter gene. The VP16-NCoRc construct did not significantly enhance the transcriptional activity of the DHT-liganded AR, and was inhibitory in some experiments (presumably due to disruption of coactivator recruitment despite the VP16 transactivation domain) (Fig. 5B). In contrast, the RU486-liganded AR was markedly coactivated by VP16-NCoRc, indicating that NCoR could be strongly recruited to an ARE by the unmodified RU486-liganded AR.

Given that these experiments employed a multimerized artificial ARE, we next asked whether NCoR could be recruited by RU486 to the physiological AREs regulating the PSA gene. As shown in Fig. 5C, RU486 functioned as a weak partial AR agonist when assayed on a luciferase reporter regulated by the PSA upstream region, containing the androgen-regulated PSA promoter and enhancer. Cotransfection with VP16-NCoRc did not substantially alter DHT-stimulated activity, but markedly enhanced the activity of the RU486-liganded AR, confirming NCoR recruitment to a physiological reporter. These results

suggested that recruitment of endogenous NCoR might contribute to the AR antagonist activity of RU486. This was tested by cotransfection with an NCoR siRNA construct. As shown in Fig. 5D, the weak agonist activity of RU486 toward the AR was enhanced by NCoR siRNA, indicating that recruitment of endogenous NCoR contributed to RU486 antagonist activity. It should be noted that the level of transcriptional activity obtained with the RU486-liganded AR and NCoR siRNA was still modest compared with the DHT-liganded AR. This modest activity is consistent with the minimal ability of the RU486-liganded AR to recruit coactivators, as assessed by SRC-1 or SRC-2 cotransfections (data not shown), and lack of N-C-terminal interaction (see below), but may also reflect recruitment of other corepressors.

AR N Terminus Is Required for NCoR Recruitment by the RU486-liganded AR—Further experiments were carried out to determine the mechanism of NCoR interaction with the RU486-liganded AR. Binding to the AR LBD was assessed using an AR DBD-LBD construct, which has minimal transcriptional activity in the absence of the N terminus on a ARE (ARE₄-Luc-Fig. 6A). The AR N terminus (expressed as a VP16-AR N-terminal fusion protein) interacted with the AR DBD-LBD in the presence of DHT, reflecting the LXXLL-like motif in the AR N terminus that interacts strongly with the agonist-liganded coactivator binding site in the AR LBD (Fig. 6A). In contrast, RU486 did not induce an interaction between the AR N terminus and LBD, consistent with the RU486-liganded AR LBD assuming a non-agonist conformation. However, despite the strong interaction between NCoR and the RU486-liganded full-length AR, the RU486-liganded AR DBD-LBD failed to interact detectably with the VP16-NCoRc protein (Fig. 6A).

Similar results were obtained in a two-hybrid protein binding assay using the AR LBD fused to the Gal4 DNA binding domain. This fusion protein interacted very strongly with the AR N terminus in the presence of DHT, but there was no detectable interaction between the RU486-liganded Gal4 AR-LBD and VP16-NCoRc (Fig. 6B). Efforts to detect such an interaction using higher concentrations of RU486 (up to 10 μ M) or of the VP16-NCoRc protein were also unsuccessful (Fig. 6B).

FIG. 7. NCoR is recruited to the AR via a specific C-terminal interaction domain. A, the indicated regions of NCoR were fused to the Gal4-DBD and cotransfected with VP16-AR in the presence of pG5-Luc and pRL-CMV. Cells were treated for 24 h with DHT or RU486 and cells were assayed as described. The data are expressed as RLU \pm S.E. B, the indicated regions of NCoR were fused to VP16 and cotransfected with the full-length AR in the presence of the ARE-Luc reporter and the pRL-CMV control. Cells were treated for 24 h with DHT or RU486, and cells were assayed as described. C, purified GST-NCoR (N2N1) *versus* GST alone were used to pull-down the indicated AR moiety that was produced in the presence of 10 nM DHT or RU486 in transfected CV1 cells. Bound AR was detected by immunoblotting with polyclonal rabbit antibody to the AR N terminus for the AR and AR N-DBD and C terminus for the AR LBD. I, 5% of input AR.

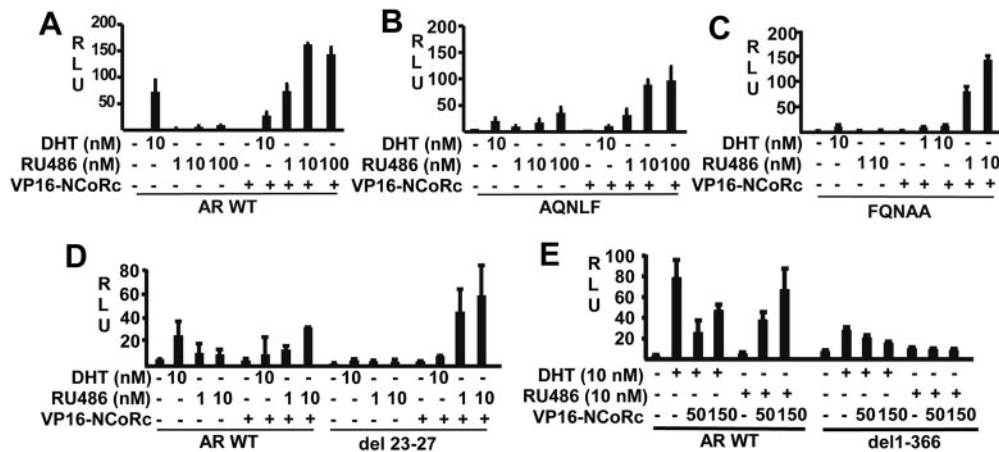
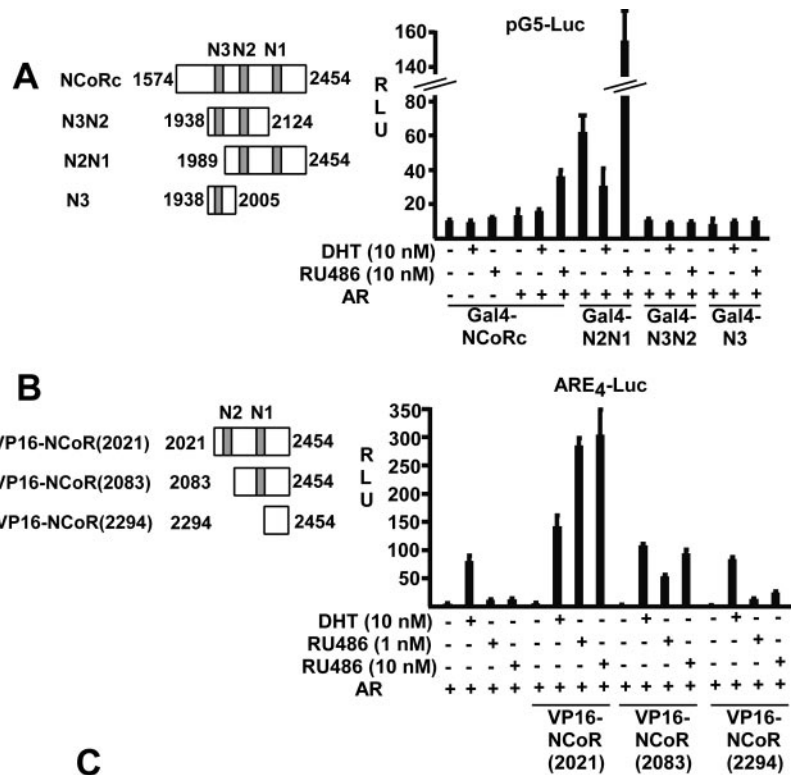


FIG. 8. AR N-C-terminal interactions are not required to recruit NCoR. The indicated AR expression vectors wild type AR (A), AR AQNLF (B), and AR FQNAA (C) were cotransfected with VP16-NCoR in the presence of ARE-Luc and pRL-CMV. Cells were treated with the indicated amounts of DHT or RU486 for 24 h and assayed for luciferase activity. The data are expressed as RLU \pm S.E. D, an identical paradigm was used to compare the wild-type AR to the deletion of amino acids 23–27 of the AR in the context of the full AR and VP16-NCoR. Cells were treated and assayed as described above. E, cells were cotransfected with the WT AR or the truncated ARdel1–366, with VP16-NCoR, in the presence of ARE-Luc and pRL-CMV. Cells were treated with 10 nM DHT or RU486 for 24 h and assayed for luciferase activity.

did not impair VP16-NCoR binding to the RU486-liganded AR. Moreover, it did not decrease the apparent high affinity for RU486 (with maximal interaction at between 1–10 nM) (Fig. 8B). The LF26,27AA double mutation, which also impairs N-C-terminal interaction and transactivation by the DHT-liganded AR, similarly failed to prevent NCoR binding by the RU486-liganded AR (Fig. 8C). Finally, deletion of all five residues (ARdel23–27) also failed to prevent AR interaction with VP16-NCoR in the presence of RU486. Taken together, these results demonstrate that the AR N terminus contributes to NCoR

binding via interactions that are distinct from those that mediate binding to the DHT-liganded AR LBD.

Previous studies have shown that the AR LBD can strongly repress a transactivation domain in the AR N terminus located between amino acids 360–528, termed transcription activation unit-5 (TAU-5), suggesting that this region may stabilize NCoR binding to the LBD (45, 46). Interestingly, an FXXLF-like motif (WHTLF) that can interact with the AR LBD is also located in this region of the N terminus (amino acids 433–437) (19). To determine whether NCoR binding was stabilized by this region

of the AR N terminus, we examined an AR(del1–366) mutant. Consistent with previous data, this mutant had substantially reduced transcriptional activity in response to DHT (Fig. 8E). However, neither the DHT or the RU486-liganded AR(del1–366) were activated by VP16-NCoR. This result indicated that NCoR binding was not responsible for repression of AR(del1–366), and that site(s) N-terminal to amino acid 366 were required for NCoR binding.

DISCUSSION

The AR plays a critical role in prostate cancer development and progression, and hormonal therapies that ablate androgen action remain as critical mainstays of therapy. However, prostate cancers invariably become refractory to androgen ablation therapies, probably through diverse mechanisms that restore AR transcriptional activity despite the presence of castrate androgen levels or currently used AR antagonists (7, 8). We and others have previously demonstrated that the corepressors NCoR and SMRT can associate with the AR and inhibit AR transcriptional activity, but the roles these corepressors play in mediating AR responses to physiological agonists or to antagonists used for prostate cancer treatment have not been determined (24–27). This study confirmed the role of endogenous NCoR as a negative regulator of AR activity, and indicated that agonist stimulated AR activity may be determined by the relative levels of NCoR *versus* coactivators. Significantly, while bicalutamide and other AR antagonists used for prostate cancer treatment could support AR recruitment of NCoR, RU486 was unique in its ability to markedly enhance the AR-NCoR interaction. This interaction was dependent on both the AR LBD and the N-terminal domain, the latter being independent of the FXXLF motif that mediates agonist-stimulated AR N-C-terminal interactions. Taken together, these results have identified a novel mechanism for AR recruitment of NCoR, which may be exploited for the development of potent AR antagonists with activity in early and potentially advanced prostate cancer.

The role of endogenous NCoR in AR action was shown by siRNA experiments, with NCoR down-regulation causing an increase in DHT stimulated AR activity. Chromatin immunoprecipitation experiments in LNCaP prostate cancer cells further demonstrated DHT-dependent NCoR recruitment *in vivo* to both the PSA and p21 promoters. NCoR associates with HDAC3, and it seems likely that histone deacetylase activity contributes to NCoR-mediated AR repression *in vivo* (47, 48). Nonetheless, we found previously that AR repression by transfected NCoR was dependent upon the C-terminal RIDs, and was not abrogated by deletion of the HDAC interacting N-terminal repressor domains. The data in this study show that an alternative mechanism of repression is through inhibition of coactivator binding to the AR N terminus. Significantly, a recent study also found DHT dependent recruitment of TBL and TBLR1 to the PSA gene (49). These proteins form a complex with NCoR and HDACs, and transcriptional activation of other nuclear receptors is linked to proteasome-mediated degradation of this complex. Therefore, these data indicate that NCoR may function in recruitment of the TBL complex to the DHT-liganded AR, and that NCoR degradation may be required for full AR activation by DHT.

Previous studies using chromatin immunoprecipitation demonstrated that NCoR could be recruited to the PSA gene by bicalutamide (31, 32). This AR antagonist is widely used for prostate cancer treatment, but has very limited efficacy in the treatment of androgen independent disease. Significantly, we found that the AR-NCoR interaction was not enhanced by bicalutamide, or by a series of other antagonists. In contrast, the AR partial agonist RU486 markedly enhanced AR recruitment of NCoR in a series of mammalian one- and two-hybrid

protein interaction experiments, using both an artificial multimerized ARE and endogenous AREs from the PSA gene. Moreover, NCoR siRNA enhanced the weak partial agonist activity of RU486, indicating that endogenous NCoR is recruited by the RU486-liganded AR and contributes to its antagonist activity. Two recent studies have similarly found NCoR recruitment by the RU486-liganded AR, and previous studies have shown that RU486 can recruit NCoR to the PR and GR (13, 28, 41–43). The unique structural feature of RU486 is a bulky group in the 11 β position, which appears to interfere with binding of coactivator LXXLL-motifs and allow for CoRNR box binding. Taken together, these studies demonstrate that the structure of the AR LBD can be altered by appropriate ligands to markedly enhance NCoR binding, and suggest that such ligands may function as more potent AR antagonists than bicalutamide or other AR antagonists currently in use for prostate cancer treatment.

Recruitment of NCoR to the DHT-liganded AR requires one or more of the three RIDs in the C terminus of NCoR, as mutations in all three RIDs abrogate NCoR repression of the DHT-liganded AR. The NCoR and SMRT RIDs share a common helical motif (LXX(H/I)XXXI), but it is clear that differences among the domains allow for nuclear receptor specificity (34, 37, 50–53). The most N-terminal of the NCoR RIDs, N3, is required to recruit the TR. This RID is not present in SMRT and explains the preference of NCoR for the TR (34, 53). The middle NCoR RID, N2 prefers the TR but can also interact weakly with the RAR. The unique CoRNR box sequence present in the homologous domain in SMRT, S2 (ISEVI), allows SMRT to preferentially recruit the RAR (36, 37). The C-terminal domains in NCoR and SMRT, N1, and S1, share significant homology and have been shown to bind to RXR isoforms strongly and to be recruited to PPAR α in the presence of antagonist (54). In this study we demonstrate that the recruitment of NCoR to the RU486-liganded AR requires the C-terminal N1 domain and is enhanced by N2, but is independent of N3. Furthermore, the interaction between N2 and N1 and the DHT- or RU486-liganded AR is mediated by a direct protein-protein interaction. These findings indicate that the AR homodimer may be similar to other nuclear receptor dimers, which recruit a single NCoR molecule via two RIDs. Preferential binding of N1 by the AR is supported by a recent study examining the recruitment of SMRT to the DHT-liganded AR, which found that the homologous S1 domain interacted most strongly in GST solution assays with the AR (26). Whether the additional preference for N2 instead of N3 is secondary to its closer proximity to N1 or to its sequence is not yet clear. Further mutational studies are underway to understand the structural mechanism by which separate NCoR RIDs are recruited to the AR, and these should aid in the rational design of new potent AR antagonists.

While the AR LBD is necessary for NCoR recruitment, our data show that the AR N terminus is also required for a strong AR-NCoR interaction. NCoR-mediated repression of the constitutive transcriptional activity of the isolated AR N-terminal domain supports a direct interaction between NCoR and the AR N terminus. This direct interaction is further supported by the ability of NCoR to directly bind to the AR N terminus in a protein-protein interaction assay. Moreover, NCoR strongly repressed AR coactivation by SRC-1 and -2, which interact primarily with the AR N-terminal domain. As an alternative to a direct interaction between NCoR and the AR N-terminal domain, NCoR binding to the AR LBD may instead be stabilized indirectly by an interaction between the AR N terminus and LBD. However, in contrast to the DHT-liganded AR LBD, the RU486-liganded AR LBD does not interact with the AR N terminus in mammalian

two-hybrid protein interaction assays. Furthermore, mutation or deletion of the FXXLF motif from the proximal AR N terminus does not impair NCoR binding to the RU486-liganded AR. Significantly, a recent study found that NCoR binding to the RU486-liganded GR was similarly dependent on the GR N terminus, and direct interactions between the AR N terminus and SMRT have been reported (25, 27, 55). Taken together, these studies indicate that a common motif in the AR and GR N termini may bind to NCoR (and possibly SMRT), and that this interaction (in conjunction with the NCoR-AR LBD interaction) is required for stable AR-NCoR binding.

In summary, the data presented in this report demonstrate that NCoR is a physiological regulator of the agonist-liganded AR, and that the relative expression of NCoR *versus* AR coactivators may in part regulate distinct AR responses to androgens in different target tissues. This study further shows that the AR-NCoR interaction is not enhanced by AR antagonists used currently for the treatment of prostate cancer, but can be markedly enhanced by RU486. NCoR interaction with the RU486-liganded AR is mediated by the AR LBD and by a site in the AR N terminus. Importantly, a further consequence of the recruitment of NCoR and the AR N terminus by the RU486-liganded AR may be to stabilize RU486 binding, accounting for the activity of RU486 in the nanomolar range *versus* the micromolar range for bicalutamide. In any case, RU486 clearly represents a new class of AR antagonists that will likely have novel activities *in vivo*. Clinical trials of RU486 or related drugs are needed to determine whether these may be more efficacious than currently available AR antagonists in the treatment of prostate cancer, particularly advanced androgen independent prostate cancer.

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